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Regulated sodium transport in the renal connecting tubule (CNT) *via* the epithelial sodium channel (ENaC)

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Abstract The aldosterone-sensitive distal nephron (ASDN) includes the late distal convoluted tubule 2, the connecting tubule (CNT) and the collecting duct. The appropriate regulation of sodium (Na^+) absorption in the ASDN is essential to precisely match urinary Na^+ excretion to dietary Na^+ intake whilst taking extra-renal Na^+ losses into account. There is increasing evidence that Na^+ transport in the CNT is of particular importance for the maintenance of body Na^+ balance and for the long-term control of extracellular fluid volume and arterial blood pressure. Na^+ transport in the CNT critically depends on the activity and abundance of the amiloride-sensitive epithelial sodium channel (ENaC) in the luminal membrane of the CNT cells. As a rate-limiting step for transepithelial Na^+ transport, ENaC is the main target of hormones (e.g. aldosterone, angiotensin II, vasopressin and insulin/insulin-like growth factor 1) to adjust transepithelial Na^+ transport in this tubular segment. In this review, we highlight the structural and functional properties of the CNT that contribute to the high Na^+ transport capacity of this segment. Moreover, we discuss some aspects of the complex pathways and molecular mechanisms involved in ENaC regulation by hormones, kinases, proteases and associated proteins that control its function. Whilst cultured cells and heterologous expression systems have greatly advanced our knowledge about some of these regulatory mechanisms, future studies will have to determine the relative importance of the various pathways in the native tubule and in particular in the CNT.

Keywords Kidney · Epithelial sodium channel · Aldosterone · Hypertension · Amiloride

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Introduction

Regulation of renal sodium (Na^+) excretion is crucial for the maintenance of extracellular salt and volume homeostasis and thus for blood pressure control. The final adjustment of renal Na^+ excretion is achieved in the aldosterone-sensitive distal nephron (ASDN) which comprises the late distal convoluted tubule 2 (DCT2), the connecting tubule (CNT) and the collecting duct (CD; see Fig. 1a). Aldosterone-sensitivity is conferred to the ASDN by the mineralocorticoid receptor (MR) and the enzyme 11-beta hydroxysteroid dehydrogenase type 2 (11βHSD2) that protects the MR from activation by glucocorticoids by rapidly degrading them to inactive metabolites. Transepithelial sodium transport in the ASDN is mediated by the epithelial sodium channel (ENaC) and the Na-K-ATPase in the luminal and basolateral plasma membrane, respectively (Fig. 1b). The function of both is tightly controlled by aldosterone and other hormones as well as by many extra- and intracellular factors.

Although the ASDN re-absorbs less than 10% of the filtered sodium (Na^+) load, the ASDN is finally decisive for the amount of Na^+ that appears in the urine [210, 246]. The ASDN is also the tubular site for net renal potassium (K^+) excretion, contributes to renal acid secretion and comprises

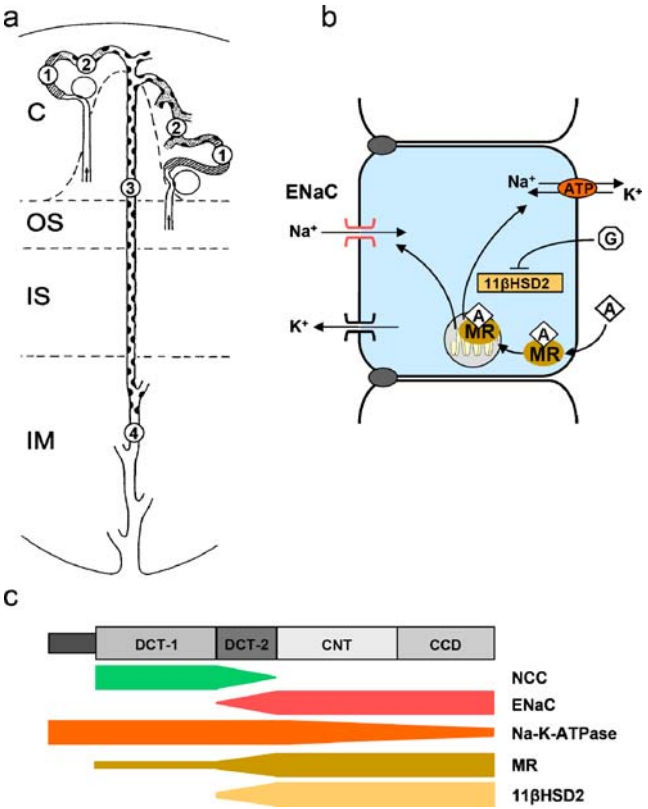

Fig. 1 The aldosterone-sensitive distal nephron. **a** Segmental organisation: The ASDN comprises the end portion of the distal convoluted tubule (*1*; i.e. the DCT2), the connecting tubule (*CNT*; *2*) and the collecting duct (*CD*) with its cortical (*CCD*; *3*) and medullary (*4*) portions. The *dark shaded areas* in the tubule represent intercalated cells that are interspersed between the segment-specific cells of the DCT2, CNT and CD. The different kidney zones are indicated as *C* cortex, *OS* outer stripe of outer medulla, *IS* inner stripe of outer medulla, *IM* inner medulla. Note the different structure of the CNT of the superficial (unbranched) and the deep nephron (one arcade with several inflows from other CNTs). **b** Simplified ASDN cell model depicting the key characteristics of the ASDN: The epithelial sodium channel (*ENaC*), the Na-K-ATPase, the mineralocorticoid receptor (*MR*) and the enzyme 11-β-hydroxysteroid dehydrogenase type 2 (*11βHSD2*) as well as an apical potassium channel. **c** Distribution of ENaC, Na-K-ATPase, MR and 11βHSD2 along the different ASDN segments in rodents. Distribution of the DCT-specific thiazide-sensitive NCC is also indicated. Changing heights of individual bars indicate axial differences in protein abundance. **a** Adapted from [157] with permission, data in **c** refer to [14, 160, 199]. For more details see text

sites for regulated transcellular magnesium and calcium reabsorption in the kidney [37, 68, 134, 246, 268, 352]. Several human tubulopathies, genetic and acquired, have been attributed to specific dysfunctions of ion transporting proteins in the ASDN [111, 194, 282]. Transgenic mouse models, mimicking human diseases, underscore the key role of the ASDN for ion homeostasis [96, 111, 143, 288]. The detailed physiology and pathophysiology of the DCT, CNT and CD as well as that of their ion transporting proteins have been discussed in depths in several recent reviews (e.g. [20, 26, 27, 47, 87, 97, 108, 111, 134, 162, 170, 187, 208, 220, 246, 254, 257, 282, 283, 285, 286, 291, 313, 318, 338] and several articles in this special issue of the journal [37, 288, 352]. Here, we will specifically focus on the mechanisms of Na^+ transport and its regulation in the CNT since evidence is emerging that this nephron segment is particularly important for the maintenance of Na^+ balance and the long-term control of arterial blood pressure [220, 246]

Histology and embryology of the CNT

The term "connecting tubule" originates from the fact that the CNT *connects* the DCT with the cortical collecting duct (CCD). The phylogenetic and embryological origin of the CNT is uncertain. A distinct tubular segment with expression of CNT-specific marker molecules is detectable already during the pronephric development in *Xenopus laevis*, indicating that the CNT evolved quite early in the evolution of the mammalian nephron, probably more than 360,000 million years ago [264]. Also during metanephric development of the rat kidney, a sharp cytogenetic border between the immature CNT and the taller and darker-stained ampullary epithelium of the collecting duct is consistently seen [234]. The sharp boundary diminishes with progressive differentiation of the CNT, but the junction between CNT and CD usually persists in the upper third of the medullary rays at the border between cortical labyrinth and medullary ray [234] as indicated in Fig. 1a. Based on these morphological observations and additional immunohistochemical criteria, investigators concluded that CNT and CD are distinct entities which originated from the nephrogenic blastema and the ureteric bud, respectively [154, 234, 244]. However, others proposed that both CNT and CD arise from the branching ureteric bud [137]. Schmitt and co-workers raised a third hypothesis suggesting that the CNT develops by mutual induction processes initiated at the border of adjoining segments [299]. This would finally lead to a unique hybrid epithelium. Consistent with this idea, the CNT of the adult kidney shares characteristics of both the upstream nephrogenic DCT and the downstream ureteric CD. Like the DCT, the CNT expresses high amounts of calcium transporting proteins in the apical and basolateral plasma membrane [14, 199]. Like the CD, the CNT has numerous intercalated cells and expresses high levels of ENaC and, in some species (e.g. rat, mouse and humans), also the vasopressin-sensitive water channel aquaporin-2 (AQP-2) [199].

The morphology and structure of the CNT is complex. Like in the collecting duct, the epithelium of the CNT is composed by two distinct cell types, namely segment-specific CNT cells and intercalated cells [81, 153, 164, 207]. The segment-

specific CNT cells are the Na^+-re-absorbing cells, whilst the intercalated cells are involved in the renal control of acid–base homeostasis (for more details on intercalated cells, see Wagner et al. [345] in this special issue). The segment-specific CNT cells reveal an ultrastructure that is intermediate between that of the typical DCT and CD principal cells [81, 155, 207]. The CNT cells have less mitochondria and basolateral plasma membrane infoldings than the DCT cells, but are considerably taller and have more mitochondria than the principal cells of the CCD [153, 155, 207]. The abundance of basolateral plasma membrane infoldings and mitochondria progressively decreases along the axis of the CNT to the CD [81, 155] consistent with axial differences of ion transport rates along the CNT and the CCD (see below) [155, 199, 207]. The transitions from DCT to CNT and from CNT to CCD are clearly demarcated only in the rabbit, whilst they are more gradual in the mouse, rat and human kidneys (reviewed in [199]). These structural differences are also reflected in some differences in the serial arrangement of ion and water transporting proteins between these species [199]. In particular, mouse, rat and human kidneys reveal at the transition from DCT to CNT a short nephron portion, in which the DCT-specific thiazide-sensitive NaCl co-transporter (NCC) overlaps with ENaC [14, 197, 299]. As this portion expresses NCC, it is considered to be part of the DCT. Bachmann and co-workers designated this late DCT portion DCT2 in distinction from the early DCT (DCT1) that is NCC-positive but ENaC negative [14, 299].

The structure of the CNT differs between nephron populations. Whilst the CNT of a superficial nephron is a rather simple tube that links one DCT with one CCD, the CNTs of mid-cortical and deep nephrons are more complex and merge to form the so-called arcades [153]. These arcades ascend in the cortical labyrinth along the cortical radial vessels before they open into the CCD in the medullary rays. The number of nephrons, which are drained by one arcade, varies amongst nephrons and amongst species [199]). A recent three-dimensional reconstruction of the mouse nephron revealed that in this species, six to seven nephrons are drained *via* the CNT to one CCD [367]. Although the individual CNT segments are rather short and in the rat have a length of about 0.5 mm [82], the contribution of the CNT to the tubular volume of the renal cortex should not be under-estimated. In the mouse kidney, the CNT accounts for more than 8% of the fractional cortical tubular volume, which is less than the fractional volume of the DCT (~12%) but clearly exceeds that of the CCD (~4%) [200]. Thus, in the CNT, the available luminal surface for sodium absorption is probably much greater than that available in the entire collecting duct.

Another morphological peculiarity that distinguishes the CNT from CD is the close apposition of the CNT to the afferent arteriole of its own glomerulus [83]. This led to the speculation that the CNT and the afferent arteriole form another site for tubuloglomerular feedback, distinct from the one located at the macula densa region. Consistent with this idea, Morsing and colleagues showed that the glomerular filtration rate rises when the tubular fluid flow is interrupted in the connecting tubule [226]. Recent studies corroborated these early observations and indicated that the glomerular feedback mechanism in the CNT depends on the sodium transport activity of this segment [269] and is coupled to the release of prostaglandins and epoxyeicosatrienoic acids [270].

Sodium transport in the CNT

Transepithelial Na^+ transport across renal epithelia is a two-step process that involves Na^+ uptake from the tubular lumen into the epithelial cells and extrusion of Na^+ across the basolateral plasma membrane into the renal interstitium from where it is taken back into the blood stream by diffusion into the peritubular capillaries. Along the ASDN, including the CNT, the luminal Na^+ entry step is represented by ENaC whilst the basolateral extrusion of Na^+ is mediated by the Na-K-ATPase [97, 162]. The activity of ENaC and the Na-K-ATPase is electrogenic and generates a transepithelial potential difference that drives K^+ secretion *via* apical K^+ channels (Fig. 1b) such as the renal outer medulla potassium channel (ROMK) and the flow-dependent maxi K (BK) channels [121, 130, 246, 352]. Whilst ROMK is exclusively present in the ENaC expressing segment-specific cells [221, 247, 361], the BK channel appears to be expressed in both the segment-specific CNT cells and the intercalated cells as indicated by immunohistochemical data [120]. However, electrophysiological techniques detected BK channel activity mainly in intercalated cells [247]. Interestingly, the intercalated cells may not only secrete but also re-absorb K^+. Intercalated cells possess an H-K-ATPase in their apical plasma membrane [94, 308, 334], which may enable these cells to transport K^+ against steep electrochemical gradients. By re-absorbing K^+, intercalated cells may represent an important cellular pathway to limit renal K^+ losses during ENaC activation. A close link between ENaC-mediated Na^+ transport and intercalated cell function can be also deduced from the intriguing observation that the first appearance of intercalated cells along the nephron coincides precisely with the onset of ENaC expression as seen in rat [299], mouse [197] and rabbit [196] kidneys. Consistent with this idea, recent studies on mice deficient for pendrin, the apical Cl^-/HCO_3^- exchanger of type B intercalated cells, pointed to a functional crosstalk between pendrin and ENaC along the ASDN [167]. Na^+ re-absorption *via* ENaC also

contributes to the osmotic gradient for vasopressin-dependent transepithelial water transport *via* the apical water channel AQP2 [16]. The importance of ENaC for Na^+, K^+ and fluid homeostasis is emphasised by the observation that gain-of-function mutations of ENaC (Liddle's syndrome) or loss-of-function mutations of ENaC (pseudohypoaldosteronism type I, PHA-I) lead to severe arterial hypertension or renal salt wasting syndromes associated with hypokalemia and hyperkalemia, respectively [162, 282]. Likewise, transgenic mouse models deficient for ENaC sub-units develop a severe PHA-I phenotype and die shortly after birth because of severe renal salt wasting with hyperkalemia (β- or γENaC deficient mice) or because of an inability to clear their lungs from alveolar fluid (αENaC-deficient mice) [288].

Molecular structure of ENaC

ENaC is a heteromultimeric channel composed of three homologous subunits (α, β, γ) with a 30% to 40% identity at the level of their amino acid sequence [50, 271]. Different techniques were used to assess the sub-unit stoichiometry of the native channel and resulted in models with either four [8, 62, 77, 100, 177] or eight to nine sub-units [90, 310]. In contrast, the recently published crystal structure of the related acid sensing ion channel acid-sensing ion channel 1 suggests that ENaC is a heterotrimer [51, 146]. However, this heterotrimeric model awaits confirmation by a crystal structure of ENaC. Each sub-unit of ENaC contains two transmembrane domains (M1 and M2), a large extra-cellular loop and short intra-cellular amino and carboxyl termini. With their M2 domains, all sub-units are thought to contribute to the channel pore [162]. Full activity of ENaC requires the co-expression of all three sub-units. Na^+ movement across the channel is highly sensitive to the diuretic amiloride (Ki~100 nM) and corresponds to the electrophysiologically measurable amiloride-sensitive Na^+ transport in native epithelia [114, 162]. In addition to the well-characterised αβγ-sub-units, a fourth ENaC sub-unit, δENaC, has been cloned from a human kidney cDNA library with transcriptional expression in a range of tissues with the highest expression levels in testis, ovary, pancreas and brain. Small amounts of δENaC-mRNA were also detected in heart, placenta, lung, liver, kidney, thymus, prostate, colon and lymphocytes but not in small intestine and spleen [347, 363]. In heterologous expression systems, δ-ENaC has functional similarities with αENaC. However, δβγ-ENaC is more than an order of magnitude less sensitive to amiloride than αβγ-ENaC [149, 150, 347]. Moreover, δβγ-ENaC but not αβγ-ENaC has been reported to be activated by extra-cellular protons [148, 363]. Another difference is the higher single-channel Na^+ conductance of δβγ-ENaC (~12 pS) compared to αβγ-ENaC (~5 pS) [347]. Interestingly, self-inhibition by extra-cellular Na^+ is less pronounced in δβγ-hENaC than in αβγ-ENaC [150]. Na^+ self-inhibition is a biophysical hallmark of ENaC and is a mechanism to prevent an intracellular Na^+ overload in transporting epithelial cells in the presence of a high extracellular Na^+ concentration [31, 114, 135, 162, 330]. Whilst the inhibitory effect of extra-cellular Na^+ on ENaC is rapid and caused by an acute decrease in channel open probability (Na^+ self inhibition) [60], the inhibitory effect of an increased intracellular Na^+ concentration is a slower process and mainly involves a decrease in channel surface expression (Na^+ feedback inhibition) [161, 175, 342]. However, recent evidence suggests that intracellular Na^+ also inhibits ENaC through an inhibitory effect on open probability [7]. It is conceivable that the sensitivity of ENaC to Na^+ feedback regulation may need to be adjusted along the nephron according to the different luminal Na^+ concentrations in different nephron segments. It is tempting to speculate that this may be achieved for example by varying the relative expression of the δ- versus the α-subunit. However, at present, it is still unclear whether the δ-subunit of ENaC is relevant for transepithelial Na^+ transport in the kidney.

Importance of the CNT for Na^+ homeostasis

The "hidden" localisation of the CNT in the cortical labyrinth and its complex structure make the CNT difficult to isolate and to study by direct functional methods [220, 246]. Nevertheless, the ion transport, electrophysiological and morphological studies performed so far indicate that ENaC activity is high in the CNT and that this segment is of major relevance for the renal control of Na^+ homeostasis. Classical micro-puncture experiments on the rat "distal convolution", which includes anatomically DCT and CNT portions, revealed that more than 90% of the Na^+ delivered to the distal convolution is re-absorbed in DCT and CNT [210] and does not even reach the downstream localised collecting duct. Moreover, micro-perfusion experiments demonstrated robust amiloride-sensitive Na^+ transport in "late" rat distal tubules (approximately DCT2 and CNT) [63], but not in collecting ducts [267, 328]. Furthermore, direct measurements of Na^+ fluxes in isolated rabbit renal tubules revealed three- to fourfold higher net Na^+ re-absorption rates in CNTs than in CCDs [4]. Likewise, transepithelial voltage differences, which are a useful indicator for ENaC activity, were reported to be more than seven times higher in the rabbit CNT than in the rabbit CCD [145]. Immunohistochemical studies corroborated these functional data by showing that the apical density of ENaC progressively decreases along the axis of the ASDN

as shown exemplary for the rabbit kidney in Fig. 2. In animals maintained on a standard laboratory diet, apical localisation of ENaC subunits is seen only in the early ASDN (i.e. the late DCT and early CNT in rat [292], mouse [197] and human [30] and the early CNT in rabbits [196]). In farther downstream portions (i.e. the middle and late portions of the CNT and the CD), ENaC resides predominantly at intracellular sites [196, 197, 214, 299]. Only the αENaC subunit was reported to have a more pronounced apical localisation that extends into the collecting duct even under standard diet [123]. The functional significance of this observation is unclear but is consistent with a non-coordinated regulation of ENaC subunits observed in cultured *X. laevis* kidney A6 cells, an *in vitro* model system for the ASDN [353]. The restriction of apical ENaC abundance to early ASDN cells in immunohistochemical studies may explain why in recent patch-clamp studies on rat CNT, ENaC whole-cell currents were undetectable in CNTs of rats maintained on a standard salt diet, whilst large ENaC whole-cell currents were observed when animals were infused with aldosterone [106]. In contrast, a recent study in mice revealed sizeable ENaC whole-cell currents in late CNTs and early CCDs of animals maintained on a standard diet. These ENaC currents further increased when animals were maintained on a low Na^+ diet [235].

Collectively, these data indicate that under standard dietary conditions, renal ENaC activity is mainly localised in the early ASDN (i.e. the late DCT and early CNT). ENaC activity in the more distal portions of the ASDN is likely to increase when Na^+ absorption needs to be maximised to maintain Na^+ balance (e.g. in response to dietary Na^+ restriction or extracellular volume depletion) [197, 220]. In fact, a variety of functional and morphological studies on rat, mouse and rabbits showed that dietary Na^+ restriction or mineralocorticoid infusion extend apical ENaC activity [67, 106, 112, 245, 267, 328] and apical ENaC immunostaining [76, 197, 202, 214, 239, 287] from the early CNT into the downstream localised ASDN portions including the collecting duct. The variation of apical ENaC immunostaining in response to altered dietary Na^+ intake is schematically presented in Fig. 3.

In addition to the effects on ENaC, prolonged elevation of plasma aldosterone does also stimulate the basolateral Na^+ transport machinery (i.e. the mitochondrial volume, basolateral membrane area and the Na-K-ATPase activity per unit tubular length; reviewed in [97, 206]). Interestingly, although the relative amount of the increase of mitochondrial density, basolateral membrane area [156] and Na-K-ATPase activity [112, 184] is most pronounced in the collecting duct, the absolute values of these parameters still remain higher in CNT than in CD [112,

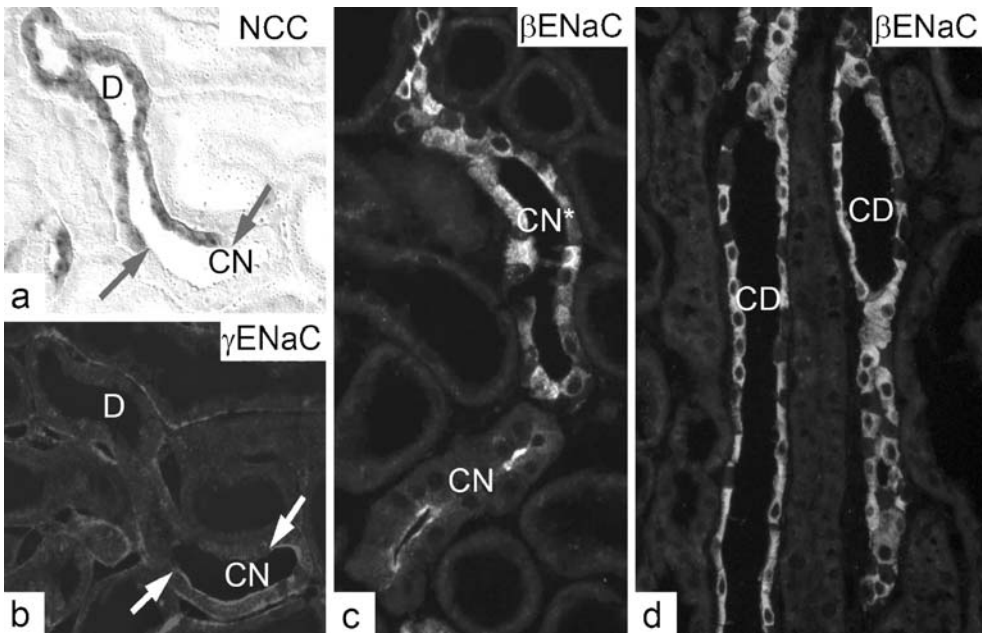

Fig. 2 Axial differences of apical ENaC localisation along the rabbit ASDN. **a** Detection of NCC mRNA by in situ hybridisation characterises the distal convoluted tubule (*D*). The NCC related signal ceases precisely at the transition (*arrows*) from D to the connecting tubule CN. **b** Immunohistochemical detection of γENaC begins precisely at the transition from D to CN and reveals a predominant apical localisation of ENaC in the early CN. **c** Immunohistochemical detection of βENaC shows also a predominant apical localisation of ENaC in the early CN whilst in farther downstream localised connecting tubules (*CN**), ENaC is mainly located at intracellular sites. **c** Likewise, collecting ducts (*CD*) show a diffuse cytoplasmic distribution of βENaC. Unstained cells in CN and CD epithelia in **c** and **d** represent intercalated cells. **a**, **b** Consecutive sections and were adapted from [196] with permission

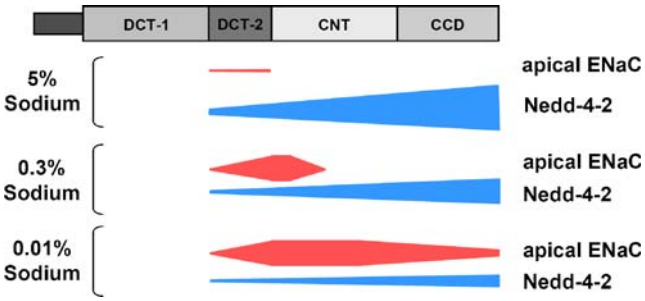

Fig. 3 Schematic presentation of axial differences of apical ENaC localisation and cytoplasmic Nedd4–2 abundances along the ASDN of mice kept for 2 weeks on a diet with high (5%), standard (0.3%) or low (0.01%) sodium content. *Heights of bars* indicate intensities of apical ENaC and cytoplasmic Nedd4–2 stainings as assessed by immunofluorescence on cryosections of mouse kidneys. Note the inverse relationship of apical ENaC and Nedd4–2 abundance along the ASDN and in response to altered dietary Na intake. Data refer to [195]

156, 184], suggesting that even under high aldosterone conditions, the Na^+ transport rates stay higher in the early ASDN portions. Consistent with these findings, immunohistochemical studies on mouse and rat kidneys as well as patch clamp studies on isolated rat renal tubules revealed a progressive decline of apical ENaC immunostaining and activity along the axis of the ASDN (CNT>CCD) even under conditions of severe Na^+ restriction [106, 197, 292]. The reason for this marked axial gradient along the ASDN is unclear. Axial differences in the expression levels of MR and 11βHSD2 are probably not involved since they appear to be similar along CNT and CD [39, 84, 92]. Moreover, in response to aldosterone, the upregulation of αENaC and of the aldosterone-dependent kinase Sgk1 (see below) occur uniformly all along the ASDN [198]. Thus, other factors appear to be responsible for the differential regulation of apical ENaC abundance and activity along the ASDN. Recently, we revealed by immunohistochemical studies that the ubiquitin-ligase Nedd4–2, which reduces the cell surface abundance of ENaC in heterologous expression systems [1, 118], shows an axial gradient along the ASDN, which is inverse to that of ENaC and therefore may explain the axial gradient of apical ENaC [195]. The observed axial gradients of apical ENaC localisation and Nedd4–2 abundance as assessed by immunostainings are schematically presented in Fig. 3.

Independent of the underlying mechanism, the aforementioned data clearly indicate that the Na^+ transport capacity of the CNT exceeds by far the one of the CD. Indeed, using whole-cell current data and considering the anatomical length of the two segments, Frindt and Palmer estimated that the Na^+ transport capacity of the CNT is at least ten times higher than that of the CD [106]. The predominant functional importance of the DCT2 and CNT for ENaC-mediated Na^+ absorption is further supported by the finding that mice with a collecting duct-specific gene inactivation of the α-subunit of ENaC are able to maintain Na^+ balance, even when challenged by salt restriction [287]. In contrast, mice with targeted deletion of the MR in the CD and late CNT but intact MR expression and ENaC regulation in early CNT and DCT2 show impaired Na^+ balance. Although the mice develop normally under standard diet, they continuously lose body weight and show signs of severe extra-cellular volume contraction under low Na^+ diet [279]. The data suggest that maintained MR-dependent ENaC regulation in DCT2 and early CNT is sufficient to keep mice in body Na^+ balance under standard conditions but not when stressed by dietary Na^+ restriction [279].

Regulation of ENaC—overview

The differences between CNT and CD with respect to Na^+ transport are more quantitative than qualitative [246, 287]. Indeed, the regulatory mechanisms controlling ENaC activity are probably quite similar along the ASDN. What differs along the axis of the ASDN is most likely the relative impact that each of these mechanisms has on ENaC regulation. ENaC is regulated by a variety of factors including hormones (e.g. aldosterone, angiotensin II, vasopressin, insulin, insulin-like growth factor I), extracellular and intra-cellular proteases (e.g. channel-activating proteases (CAP1-3), tissue kallikrein, furin), intra- and extracellular ion concentrations, osmolarity, tubular flow rate, as well as kinases (e.g. Sgk1, protein kinase A (PKA), extracellular-regulated kinase (ERK)) and interacting proteins (e.g. ubiquitin ligases, deubiquitinylating proteases, Rab proteins) which have been discussed in detail in several recent reviews [20, 26, 27, 47, 87, 114, 142, 162, 169, 182, 187, 201, 208, 254, 260, 283, 285, 302, 313, 318, 322, 338]. We have not attempted to provide a comprehensive review of this vast and rapidly expanding field of research on ENaC regulation. Instead, we have limited our discussion in the following to some selected aspects of ENaC regulation thought to be relevant in the kidney and the CNT in vivo.

Regulation of ENaC by aldosterone

Aldosterone is the main hormonal regulator of ENaC-dependent Na^+ transport in the distal nephron [114, 162, 337]. Aldosterone binds to the intracellular MR, which upon translocation to the cell nucleus induces or represses the transcription of genes encoding proteins of the Na^+-transporting machinery (e.g. ENaC, Na-K-ATPase) and proteins that regulate the activity of the Na^+-transporting apparatus [109, 110]. A variety of *in vitro* and *in vivo* experiments

established that aldosterone specifically increases the synthesis of αENaC in ASDN cells, whereas β- and γENaC are constitutively expressed (reviewed in [114, 162]). Changes in plasma aldosterone levels, either induced by dietary Na^+ restriction or by exogenous application of the hormone, induce a redistribution of ENaC subunits from intracellular compartments to the apical plasma membrane [76, 197, 198, 214]. The activation and apical translocation of ENaC occurs quite rapidly (within hours) and is probably relevant for the renal adaptation to day-to-day variations of Na^+ intake [104, 105, 198]. The observed apical recruitment of ENaC goes along with the appearance of low molecular weight forms of α- and γENaC subunits, which are thought to reflect increased proteolytic cleavage and activation of the channel [104, 214]. Recently, Frindt and co-workers performed cell surface biotinylation experiments on rat kidneys perfused with biotin *in situ*. These experiments showed that feeding a low Na^+ diet or the infusion of aldosterone for 1 week increases the cell surface pool of ENaC subunits by two- to fivefold. The experiments also indicated that in contrast to βENaC, most of the α- and γENaC subunits at the cell surface are proteolytically cleaved [107].

Based on previous studies in *Xenopus laevis* A6 cells, it has been proposed that the induction of αENaC might be a prerequisite for full assembly of ENaC in the ER and its subsequent delivery to the cell surface [217]. However, in the kidney in vivo, the induction of αENaC protein by aldosterone is rather small [214] and, at least in the CCD, not necessarily followed by an apical re-distribution of all three ENaC subunits [198]. Moreover, apical targeting of ENaC apparently occurs even without previous αENaC induction [238]. Thus, the induction of αENaC alone cannot account for the apical targeting of ENaC. Other co-stimulatory factors are needed.

The Sgk1 kinase (serum- and glucocorticoid-regulated kinase), a member of the PKB/Akt family of serine/threonine kinases, has been identified as one of the aldosterone-induced regulatory proteins that impacts on ENaC cell surface expression and activity [182, 187, 201, 249]. Sgk1 is rapidly induced by aldosterone in ASDN model epithelia in vitro as well as in the kidney in vivo [57, 198, 232, 306]. Likewise, prolonged dietary Na^+ restriction increases Sgk1 mRNA expression in the kidney [136]. Co-expression of ENaC with Sgk1 in heterologous expression systems profoundly increases ENaC-mediated Na^+ currents probably by an accelerated insertion rate of ENaC into the plasma membrane [5]. The channel open probability might be increased as well [344], perhaps by direct phosphorylation of a specific serine residue in the C terminus of the channel's α-subunit [74]. The ubiquitin-ligase Nedd4–2 has been proposed to mediate at least some of the effects of Sgk1 on ENaC cell surface abundance. In heterologous expression systems, Sgk1-dependent phosphorylation of Nedd4–2 inhibits Nedd4–2 interaction with a proline-rich (PY)-motif in the C terminus of ENaC subunits and thereby presumably prevents Nedd4–2-induced ubiquitylation with subsequent endocytosis and degradation of ENaC subunits [70, 265, 311]. In mouse mpkCCD cells in vitro as well as in rat CCD in vivo aldosterone rapidly induces the phosphorylation of Nedd4–2 that precedes the activation and increased cell surface abundance of ENaC [101]. However, the observed effects are rather small and Nedd4–2 is significantly phosphorylated even in the absence of any aldosterone and aldosterone-induced Sgk1 [101] suggesting that other (possibly aldosterone-independent) kinases may contribute to the control of Nedd4–2 phosphorylation and activity. In fact, other kinases such as PKB/Akt and PKA were reported to phosphorylate Nedd4–2 as well [186, 312].

In addition to the rapid effects of aldosterone on Nedd4–2 phosphorylation, aldosterone may have chronic effects on Nedd4–2 protein levels, which might be relevant for the long-term adaptation of the ASDN to altered dietary Na^+ intake. In the mpkCCD cell line, prolonged aldosterone treatment was shown to reduce Nedd4–2 protein abundance. The decrease became clearly apparent at day2 of aldosterone treatment and was most obvious at day 6 [195]. Likewise, in mice, dietary Na^+ restriction for 2 weeks reduced Nedd4–2 protein abundance (as assessed by immunohistochemical detection) along the ASDN [195]. Taken together, the data suggest a model in which the rapid effects of aldosterone on ENaC are mediated by altered Nedd4–2 phosphorylation whilst the more chronic effects (days) are related to changes in the abundance of Nedd4–2 protein.

Regulation of ENaC by angiotensin II

Extracellular volume depletion activates the renin–angiotensin–aldosterone system which in turn increases renal Na^+ and fluid retention to restore extracellular volume homeostasis and to maintain arterial blood pressure. Independent from its effect on adrenal aldosterone secretion, angiotensin II (AngII) directly stimulates amiloride-sensitive Na^+ re-absorption in late distal tubules [351] and in collecting ducts [251]. The effect is most pronounced when AngII is applied to the luminal side of the tubules and can be blocked by the AT(1) receptor inhibitors candesartan or losartan [251]. Consistent with a direct effect of AngII on ENaC-mediated Na^+ re-absorption, AngII binding sites and AT1 receptors were revealed by biochemical [227] and immunochemical [129] methods in distal tubules and the collecting duct. The demonstration of renin expression in the CNT and the finding that its expression is regulated by dietary Na^+ intake prompted Rohrwasser et al. to propose a

paracine tubular renin–angiotensin system that may regulate ENaC function from the tubular lumen [278]. The rapid effects of AngII are likely related to the regulation of already pre-existing channels. However, there is increasing evidence that AngII may also impact on the synthesis rate of ENaC channels and that these effects are also independent from aldosterone. AT1 receptor knockout mice express less αENaC despite elevated plasma aldosterone levels [42]. Likewise, in NaCl-restricted rats, 2 days of AT1 receptor inhibition reduced αENaC mRNA and protein expression. This effect was independent from an aldosterone-dependent activation of the mineralocorticoid receptor. The reduced expression of αENaC was accompanied by a lowered density of ENaC in the apical membrane of the renal collecting system. Conversely, long-term systemic infusion of AngII induced αENaC expression in rat kidney cortex [23]. Moreover, AT1 receptor-dependent activation of ENaC was linked to the inappropriate renal Na^+ retention in diseases such as type 2 diabetes and obesity [205]. Taken together, the studies suggest that extracellular volume contraction, as it occurs during dietary Na^+ restriction, stimulates ENaC by both increased plasma aldosterone levels and enhanced AngII levels in plasma and urine.

Regulation of ENaC by vasopressin

Anti-diuretic hormone (ADH, vasopressin) enhances Na^+ absorption in *Xenopus laevis* kidney A6 cells [335] and in isolated rat cortical collecting ducts [298]. The effect of vasopressin is synergistic to that of aldosterone but occurs much more rapid [298, 335]. Vasopressin acts by binding to V2 receptors with subsequent activation of the adenylate cyclase. Its effect on Na^+ transport is mimicked by membrane permeable cAMP analogues, phosphodiesterase inhibition with 3-isobutyl-l-methylxanthine or adenylate cyclase activation with forskolin. In the kidney, V2 receptors and vasopressin-induced cAMP generation [223] were found in both the collecting duct and the CNT of rat [96, 228], mouse [228] and human [228], suggesting that in these species, ENaC regulation by vasopressin may occur in both segments of the ASDN. Chronic stimulation of the V2 receptor was reported to reduce Na^+ excretion in healthy humans further indicating that the cAMP pathway of ENaC regulation is relevant for Na^+ homeostasis in humans [16].

Functional studies on *Xenopus laevis* kidney A6 cells demonstrated that the acute activation of Na^+ channels by vasopressin depends on intact microtubules [336] and Golgi apparatus [168] suggesting that the vasopressin-induced activation of ENaC is caused by enhanced trafficking of channels from an intra-cellular pool to the apical plasma membrane. Using a combination of short circuit current (I_{SC}) measurements and antibody detection of epitope tagged ENaC, Morris and co-workers provided quantitative evidence that in MDCK cells stably expressing ENaC the increase in I_{SC} produced by cAMP can be accounted for entirely by a proportional increase in the surface density of ENaC [225]. Cell surface biotinylation experiments in mpkCCD cells with endogenous ENaC expression further confirmed the model that the acute ENaC stimulation by cAMP is mediated by exocytic insertion from a recycling channel pool [44]. However, in spite of all these convincing in vitro data, direct evidence that vasopressin increases the cell surface abundance of ENaC in the ASDN in vivo is still lacking.

The rapid effects of vasopressin on ENaC need to be distinguished from the more chronic long-term effects of the hormone that involve transcriptional effects on ENaC and ENaC regulatory proteins. Long-term treatment with vasopressin or its synthetic analogue 1-desamino-8-D-arginine vasopressin (DDAVP) markedly increases the expression of β- and γENaC sub-units in a rat cortical CCD cell line [79] and in rat kidneys [86, 236]. This effect was accompanied by a significant increase of Na^+ transport in the cultured CCD cells [79] as well as in the isolated perfused collecting ducts [79]. However, under this long-term stimulation, the induced Na^+ transport activity was probably not related to an enhanced trafficking of channel molecules to the cell surface. Immunohistochemistry revealed that the induction of β- and γENaC led to an intracellular accumulation of the channel subunits in CNT and CD of the DDAVP-treated rats, but did not go along with any detectable change in the cell surface abundance of α-, β- or γENaC [292].

Using serial analysis of gene expression, Firsov and colleagues aimed at identifying mRNAs that are rapidly up- or downregulated by vasopressin and could mediate transcriptional effects on ENaC activity. In mpkCCD collecting duct cells, treated for 4 h with vasopressin, 48 genes were induced whilst 11 genes were repressed [277]. Subsequent functional analysis indicated that two of the upregulated transcripts (vasopressin-induced transcript 32 and the regulator of G protein signalling 2) are actually involved in negative feedback regulation of V2 receptor signalling that possibly limits ENaC activation under chronic vasopressin stimulation [237, 371]. Another induced transcript, the ubiquitin-specific protease 10 (Usp10), was recently shown to increase the cell surface expression of ENaC, when co-expressed with the channel in HEK-293 cells [41] (see below).

Regulation of ENaC by insulin/IGF-1

Insulin stimulates renal Na^+ retention and K^+ excretion in man and in laboratory animals [71]. A large proportion of the response can be reversed by the ENaC blocker benzamil

[314, 325]. Insulin and insulin-like growth factor-1 receptors (both can bind insulin and insulin-like growth factor I (IGF-1), though with different affinities) have been demonstrated along the mammalian renal tubular system including the cortical collecting system [71, 124] indicating that both hormones may regulate ENaC in renal epithelia. In fact, in amphibian model systems (i.e. toad urinary bladder, *Xenopus laevis* A6 cells), insulin and IGF-1 profoundly activate amiloride-sensitive Na^+ transport by activation of basolateral and/or luminal receptors [32–34, 216, 307, 369]. Both hormones do also activate ENaC in mammalian CCD cell lines [116, 317], but the responsiveness of the mammalian CCD cells appear to be higher for IGF-1 than for insulin [116].

Experiments in *Xenopus laevis* A6 cells, mouse CCD cells and isolated collecting ducts indicated that insulin and/or IGF-1 stimulate ENaC activity by increasing the cell surface density of ENaC [18, 34, 35] and/or by increasing the open probability of Na^+ channels already present in the apical plasma membrane [213, 317]. Part of these effects might be related to direct phosphorylation of channel subunits [307, 369]. Although insulin and IGF-1 signalling has been studied in various target tissues [85, 88], only little is known about the insulin-signalling pathway in the ASDN *in vivo*. Data from in vitro assays, amphibian and mammalian renal cell-lines indicated that insulin and IGF-1 stimulate Na^+ transport via a signalling cascade that involves sequential phosphorylation and activation of phosphoinositide 3-kinase (PI3K), 3-phosphoinositide-dependent protein kinase (PDK1) and finally, Sgk1 [182, 187, 201, 249]. IGF-1 was also reported to stimulate Sgk1 expression at the mRNA and protein level [116]. This suggests Sgk1 as a central point of convergence for both the aldosterone (induction of Sgk1) and the insulin/IGF-1 (induction and activation of Sgk1) signalling pathways [182, 187, 201, 249]. Support for this hypothesis comes from experiments in A6 cells in which pharmacological inhibition of PI3K blunts hormone-induced Sgk1 phosphorylation and insulin- as well as aldosterone-stimulated Na^+ transport [348]. The rather mild renal salt wasting phenotype of Sgk1 deficient-mice [358], however, points to a certain degree of redundancy in the Sgk1-dependent signalling pathway. In this context, the putative roles of closely related PKB/Akt kinases warrant further attention [201]. These insulin-dependent kinases phosphorylate similar phosphorylation sites as Sgk1 [172]. Recent data in Fisher rat thyroid cells with heterologous expression of ENaC indicated that PKB isoforms could also mediate the effects of insulin on ENaC [186]. However, data from the *Xenopus laevis* oocyte expression system and *Xenopus laevis* kidney A6 cells did not support this concept [11]. Perhaps, cell type specific differences may explain the discrepant results. Therefore, additional experiments in mammalian renal epithelia are needed to further address this issue.

Regulation of ENaC by insulin and IGF-1 may have important clinical relevance. For example, it has been proposed that the chronic hyperinsulinaemia in type-2 diabetes inappropriately stimulates ENaC-dependent renal Na^+ re-absorption and hence may contribute to the frequent association of diabetes and hypertension in metabolic syndrome [18]. Consistent with this hypothesis, enhanced renal expression of Sgk1 and other ENaC-activating kinases have been found in kidneys of diabetic mice [178] and humans [181]. Moreover, Sgk1-deficent mice appear to be protected from the development of salt-sensitive hypertension in two models of hyperinsulinaemia [138, 139]. However, the potential role of hyperinsulinaemic activation of ENaC should not be over-estimated. Although changes in ENaC protein expression have been reported in kidneys of diabetic rodents [28, 29, 87, 205, 272–275], the functional significance of these findings is uncertain and a direct demonstration of insulin-induced ENaC hyperactivity in CNT and CD of diabetic kidneys is lacking so far. In fact, the observation that chronic hyperinsulinaemia in patients with insulinoma is not associated with a detectable elevation of blood pressure [293, 339] argues against a direct role of hyperinsulinaemia in hypertension. The observation that the insulin receptor is actually down-regulated in kidneys of hyperinsulinaemic rats [324] and that kidney-specific deletion of the insulin receptor increases renal Na^+ retention and blood pressure [326] stress that the pathogenesis of arterial hypertension in type-2 diabetes is complex.

IGF-1 has also been linked to renal Na^+ retention. IGF-1 production is under strict control of growth hormone (GH). GH infusion to healthy humans increases renal IGF-1 production and stimulates renal Na^+ and fluid transport [125, 133, 222] which has been used to explain that patients with acromegaly (GH over-production) frequently develop arterial hypertension [36]. Recently, Kamenicky and co-workers studied acromegalic GC rats to analyse the molecular mechanism responsible for renal Na^+ retention in these rats. Despite suppressed plasma aldosterone levels, the GC rats revealed an increased amiloride-sensitive natriuresis, an enhanced Na-K-ATPase activity in collecting ducts and an altered proteolytic cleavage of ENaC subunits, suggesting that enhanced ENaC-mediated Na^+ transport in the ASDN contributes to the pathogenesis of Na^+ retention in acromegaly [158]. Interestingly, genetic analysis suggested also a strong linkage between the IGF-1 gene locus and systolic blood pressure [231]. The observation that patients with nephrotic syndrome do also have excessive amounts of IGF-1 in their urine led to the speculation that urinary IGF-1 may activate ENaC-dependent Na^+ transport in the kidney and hence explain the Na^+ and fluid retention in the disease [350]. In fact, increased Na^+ re-absorption in the renal collecting system is

a hallmark of nephrotic syndrome [72, 165, 166] and appears to be initiated independent of increased plasma aldosterone [69, 201].

Regulation of ENaC by proteases

Recent evidence indicates that proteolytic processing of ENaC along its biosynthetic pathway and at the cell surface is an important mechanism that contributes to ENaC activation in a complex manner. Several excellent reviews exist on this rapidly expanding field of research [169, 254, 281, 283]. Within the scope of the present review, we can only highlight some aspects of this interesting feature of ENaC regulation.

The observation that the protease inhibitor aprotinin induced a decrease in short-circuit current in the toad bladder [243] was probably the first indication for an activating effect of proteases on ENaC. Subsequently, CAPs have been identified using the *Xenopus laevis* oocyte expression system and were shown to activate ENaC when co-expressed with the channel [331, 332, 344]. Electrophysiological studies in the *Xenopus laevis* oocyte expression system clearly demonstrated a large and rapid stimulatory effect of extracellularly applied trypsin and chymotrypsin on ENaC activity [59]. Membrane-bound and/or secreted proteases such as CAPs are likely to exist in ENaC expressing epithelia and are thought to act on the extra-cellular domain of the channel. One possible candidate for an endogenous ENaC-activating protease is prostasin, the mammalian homologue of *Xenopus* CAP1 [343]. Prostasin is an attractive candidate since it is expressed in the kidney and in cultured collecting duct cells [2, 241] and its expression seems to be regulated by aldosterone [233], the main hormonal regulator of ENaC. However, prostasin has a pH optimum of about 9 and is practically inactive at physiological urine pH [365]. Moreover, although the serine protease inhibitor aprotinin abolishes prostasin-induced activation of ENaC [2], mutations within the catalytic triad of prostasin (mCAP1) do not prevent its stimulatory effect on ENaC currents in co-expression experiments [9, 43]. In contrast, mutating the catalytic triad of the related proteases mCAP2 and mCAP3 abolishes their stimulatory effect on ENaC [9]. These findings suggest that prostasin may act indirectly by altering the activity of downstream proteases. Thus, more than one protease is probably involved in ENaC regulation. Another candidate is tissue kallikrein which recently has been reported to be involved in ENaC processing in the kidney [252]. Tissue kallikrein is synthesised in large amounts in the CNT [17, 98, 262] and released into the urine [190, 212] from where it could act on ENaC either directly or indirectly by activating other proteases [252]. Remarkably, kallikrein synthesis in the CNT and its subsequent release into the urine are stimulated by aldosterone [211], dietary Na^+ restriction [242, 300] and in particular dietary K^+ loading [122, 341] indicating that kallikrein could be involved in ENaC regulation under these conditions. Interestingly, kallikrein failed to activate ENaC heterologously expressed in *Xenopus laevis* oocytes [59]. Moreover, renal ENaC cleavage is preserved in kallikrein-deficient mice, and these animals do not develop a salt-losing phenotype even when stressed by dietary Na^+ restriction [252]. However, these findings do not rule out a functional role of kallikrein in ENaC regulation, but support the concept that ENaC activation by proteases is a highly redundant process. Indeed, in analogy to known kinase/phosphatase networks, the channel-activating proteases may be part of a complex and highly regulated protease cascade with tissue-specific properties [281, 283]. The recent discovery of endogenous CAP inhibitors adds to the complexity of this regulatory pathway [346].

ENaC function appears to be regulated also by intracellular proteases. Proteolytic cleavage by furin [323] or other Golgi-associated convertases are thought to be important for ENaC maturation in the biosynthetic pathway before the channel reaches the plasma membrane [128]. Western blot analysis of a range of ENaC expressing cells and tissues has revealed the presence of distinct ENaC cleavage products in particular of the α- and γ-subunit [283]. The channel is thought to be in its mature and active form in its cleaved state, but there is evidence for the presence of both cleaved and non-cleaved channels in the plasma membrane [141].

At present, the precise molecular mechanism of proteolytic channel activation remains unclear. Cleavage occurs at specific sites within the extra-cellular loops of the α- and γ-sub-units but not the β-subunit [3, 75, 142, 254, 283]. Cleavage at these sites probably results in the release of inhibitory peptides from the extra-cellular loops of α- and γENaC [43, 53, 55]. Cleavage sites for furin [140], prostasin [43], plasmin [248] and elastase [3] have been identified and were studied in heterologous expression systems. Mutational analysis indicated that similar stimulatory effects can be achieved by cleaving the channel at different but closely adjacent sites within a functionally important region. In particular, cleavage of the γ-subunit appears to play a pivotal role in proteolytic ENaC activation [3, 54, 75, 127]. At least two functionally distinct ENaC populations are present in the plasma membrane: active channels with a somewhat variable but rather high open probability (P_O) of about 0.5 [114] and near-silent channels with an exceedingly low P_O of less than 0.05 [99, 162, 280]. Proteolytic cleavage may cause a conformational change of the channel favouring its open state. In fact, extracellularly applied trypsin appears to have a dual

function on ENaC open probability. It activates near-silent channels [48, 49, 75] and stimulates the gating of channels that are already active in the membrane [75]. Relieving Na^+ self-inhibition may also add to the stimulatory effect of proteases on ENaC [31, 60, 301]. In addition, indirect mechanisms involving the downstream activation of G-proteins have been reported to contribute to ENaC activation by trypsin [19].

Most of our knowledge about ENaC activation by extracellular proteases stems from studies in model system like *Xenopus laevis* oocytes and cultured cells including renal epithelial cell lines. Nevertheless, the consistent observation of low molecular weight forms of γENaC and αENaC in the kidneys of rodents with elevated plasma aldosterone levels (e.g. [89, 104, 170, 214] indicated that proteolytic cleavage of ENaC occurs in native tissue. Importantly, the appearance of cleaved products correlated with increasing ENaC currents [89, 107]. Moreover, functional evidence for ENaC activation by extracellular proteases in renal tubules is emerging. In trypsin-treated isolated perfused rabbit, CCDs net Na^+ absorption significantly exceeded that measured in control tubules by about 70% [224]. Moreover, application of kallikrein elicits an amiloride-sensitive increase in the intracellular Na^+ concentration in principal cells of isolated and micro-perfused mouse CCDs [252]. Finally, whole-cell patch clamp recordings demonstrated that application of trypsin can stimulate the amiloride-sensitive current in principal cells of micro-dissected split open rat [107] and mouse [235] distal tubules. Collectively, these studies provide proof of principle that extra-cellular serine proteases can stimulate ENaC activity in native renal tissue. Functionally, the process of ENaC activation by extracellular proteases may provide a mechanism for the fast adaptation of ENaC function to altered functional requirements. Thus, a rapid activation of the so-called near-silent channels [49] already present in the luminal membrane may precede other regulatory mechanism like the biosynthesis and insertion of additional pre-existing or newly synthesised channels in the apical plasma membrane. The predominant apical localisation of ENaC in the (early) CNT makes the CNT particular susceptible for this type of regulation.

In addition to their physiological function, proteases may also be involved in the pathogenesis of renal Na^+ retention in renal diseases. Patients with crescentic glomerulonephritis have significantly higher urinary concentrations of neutrophil elastase, than healthy controls [240]. Likewise, plasminogen appears in the urine of patients with nephrotic syndrome [182, 333]. After conversion to plasmin by tubular urokinase-type plasminogen activator [253], the generated plasmin may contribute to ENaC activation [248, 320]. Indeed, the urine from puromycin aminonucleoside (PAN)-nephrotic rats has recently been shown to contain plasmin and to activate ENaC [320]. In contrast, urine from control rats or heat-inactivated urine from PAN-nephrotic rats had no stimulatory effect [320]. Whether the increased proteolytic cleavage of ENaC contributes to renal Na^+ retention in nephrotic syndrome warrants future analysis. The concentration of proteases may also be increased in the urine of diabetic patients [321] which is supported by a recent report that plasmin is increased in the urine of obese, diabetic and hypertensive ZSF1 rats [248]. Thus, it is tempting to speculate that an inappropriate ENaC activation by elevated levels of urinary proteases may contribute to arterial hypertension associated with diabetes mellitus and metabolic syndrome. Moreover, renal disease may not only affect the activity of proteases but may also shift the balance between endogenous proteases and protease inhibitors. In this context, it is of interest that serpinh1, a serine protease inhibitor, has recently been identified in an integrated genomic–transcriptomic approach as a hypertension-related gene [362]. Thus, the pathophysiological aspects of proteolytic ENaC regulation await further investigation and the identification of relevant proteases and protease inhibitors along the nephron and in urine samples in various disease states. In the future, specific protease inhibitors may become valuable therapeutic tools to prevent excessive ENaC activation under certain patho-physiological conditions. Moreover, the relative importance of intra- versus extracellular proteolytic processing of ENaC remains to be determined and may vary in different tissues and nephron segments under different (patho-) physiological conditions.

Regulation of ENaC by kinases

ENaC phosphorylation by kinases has long been thought to contribute to ENaC regulation [114]. In cultured renal epithelial cells, aldosterone and insulin have been shown to increase the phosphorylation of the COOH terminal ends of the α-, β- and γ-subunits of ENaC [307, 369]. Moreover, the COOH termini of ENaC subunits were found to be phosphorylated by cytosolic fractions derived from rat colon [58]. This phosphorylation is thought to involve at least three different types of kinases, including the ERK and the casein kinase 2 (CK-2) [303, 304].

Since these initial observations, several kinases have been identified to control ENaC function either by direct phosphorylation of ENaC subunits or by phosphorylation of proteins interacting and regulating ENaC. Perhaps the best studied kinase in this context is the aldosterone-induced kinase Sgk1 (see above) which stimulates cell surface activity and density of ENaC via phosphorylation of αENaC (increasing Po) [74] and Nedd4–2 (increasing N) [187, 201, 249], respectively. Moreover, Sgk1 may stimulate ENaC function via direct inhibition of the Dot1a–Af9

repressor complex that controls the transcription of αENaC and possibly other aldosterone-induced genes [368]. The *in vivo* significance of Sgk1 for ENaC regulation has been confirmed in several studies on two different Sgk1-deficient mouse models which both exhibit a salt-losing phenotype [95, 358]. However, only one of the studies attributed the salt losing phenotype to a reduced ENaC activity in the CCD [358], whilst the other study did not find any effect on the overall channel activity although the proteolytic cleavage of γENaC was diminished [95]. In addition to Sgk1, two Sgk1 paralogues (Sgk2 and Sgk3) have been identified [172]. These kinases have the same consensus motif (RXRXX(S/T)) for phosphorylation as Sgk1 [172] and both profoundly stimulate amiloride-sensitive Na^+ currents when co-expressed with ENaC in heterologous expression systems [103]. However, Sgk2 and Sgk3 are not regulated by corticosteroids [172] and Sgk3-deficient mice do not have renal salt wasting indicating that at least Sgk3 is not involved in ENaC regulation in the kidney [119, 218].

Other kinases thought to interfere with the Nedd4–2/ENaC pathway via direct phosphorylation of ENaC sub-units are ERK [303], CK-2 [304] and the G-protein-coupled receptor kinase GRK2 [78]. Phosphorylation of the COOH-termini of β- and γENaC by ERK and CK-2 appears to reduce ENaC cell surface density and activity by increasing the affinity of Nedd4–2 to ENaC [303, 304]. Notably, the activation of ERK can be blocked by the glucocorticoid-induced leucine zipper protein (GILZ) [26, 315, 316]. GILZ is rapidly induced by aldosterone in CCD cells *in vitro* [277] suggesting that aldosterone may regulate the Nedd4–2/ENaC interaction at two different levels: (a) by phosphorylation of Nedd4–2 (via Sgk1) and (b) by inhibition of ERK-dependent ENaC phosphorylation and subsequent Nedd4–2 binding (via GILZ). In contrast to the action of ERK and CK-2, the phosphorylation of the COOH-terminal tail of βENaC by GRK2 appears to enhance the channel activity by disruption of the Nedd4–2/ENaC interaction [78]. The observed stimulatory action of GRK2 on ENaC was proposed to explain the reported association of GRK2 over-activity with hypertension [78]. The cAMP-activated PKA is another kinase that may directly phosphorylate ENaC [307]. Although definitive proof for this has not yet emerged, Yang and co-workers recently showed that two putative ERK consensus motifs in the C termini of rat β- and γENaC are critically involved in the regulatory pathway, by which cAMP activates the channel [364]. PKA was also suggested to increase both the phosphorylation of Sgk1 [250] and Nedd4–2 [312] further supporting the idea that the aldosterone and vasopressin signalling pathway converge in ENaC regulation. Moreover, vasopressin and cAMP may affect ENaC via the recently identified exchange protein directly activated by cAMP (Epac) pathway [38]. Epac1 and Epac2 are highly abundant in the ASDN [191].

Several other kinases have been suggested to regulate ENaC function independent from direct ENaC phosphorylation. One of these kinases is the with-no-lysine (K)-kinase WNK1. WNK1 is highly expressed in the kidney and is implicated in the pathogenesis of Gordon's syndrome, a severe form of arterial hypertension with hyperkalemia, which is thought to be related to a hyperactivity of the thiazide-sensitive NCC in the DCT [152]. Recent studies suggested that WNK1 may also activate Sgk1 by a phosphatidylinositol 3-kinase-dependent non-catalytic mechanism, which finally increases ENaC activity and possibly contributes to the hypertensive phenotype [359, 360]. In addition, IκB kinase-β [185], the phosphatidylinositol 3-kinase [317] and protein kinase Cδ [219] were recently shown to activate ENaC. However, kinases may not only increase but also inhibit ENaC function. Recent evidence suggests that the metabolic sensor AMP-activated kinase may limit ENaC activity under metabolic stress when the intra-cellular ATP/AMP ratio is shifted to AMP [25, 52, 357] and may prevent cellular Na^+ loading when ATP supply to the Na-K-ATPase is limited. Pharmacological 5′AMP-activated protein kinase (AMPK) activation or over-expression of an activating AMPK mutant in a mouse collecting duct cell line inhibited amiloride-sensitive short circuit currents [52]. Additional experiments in heterologous expression systems indicated that the AMPK activation promotes Nedd4–2-dependent ENaC retrieval from the plasma membrane by mechanisms that are independent from Sgk1, PKA or ERK activation and may be caused by ENaC phosphorylation [25].

Thus, a variety of kinases involved in regulation of ENaC have been identified mainly in *in vitro* systems. The significance of these kinases for regulation of the channel in the kidney *in vivo* warrants further investigation and it remains to be established whether axial heterogeneity of their expression and activity may contribute to a differential regulation of ENaC along the nephron. To understand this, it will be important to determine the kinases and phosphatases involved in ENaC regulation in the different nephron segments. It would not be surprising to find CNT specific mechanisms for ENaC regulation by phosphorylation and dephosphorylation processes which may explain the different regulation of ENaC activity in this nephron segment compared to the collecting duct.

Regulation of ENaC function and trafficking by associated proteins

It is an emerging paradigm that membrane transport proteins do not function in isolation but interact with associated regulatory proteins which modulate channel function and trafficking (Fig. 4). Sphingolipid- and

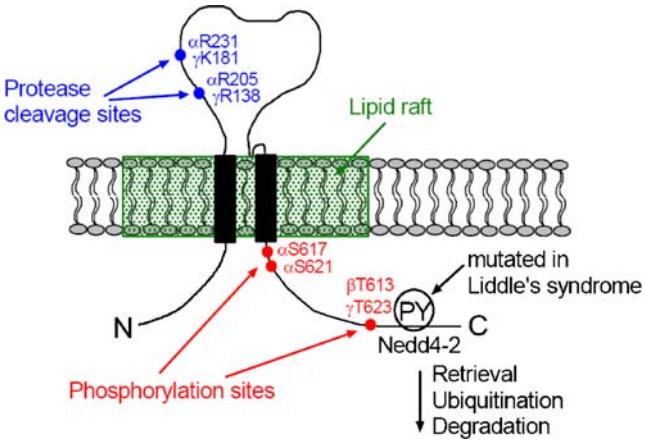

Fig. 4 Molecular mechanisms of ENaC regulation. For simplicity, only one channel subunit is shown. Putative phosphorylation and proteolytic cleavage sites in the three channel subunits (αβγ) are indicated with amino acid positions corresponding to the rat sequence. Proteolytic processing occurs at two putative furin cleavage sites in αENaC and at one furin site and one prostasin site (γK181) in γENaC. In the γ-subunit of human and mouse ENaC, additional elastase and plasmin cleavage sites have recently been identified and are localised distal to the prostasin cleavage site (not shown). Proteolytic cleavage is thought to result in the release of inhibitory peptide domains. The C-terminal PY motif (PPXY) is mutated in patients with Liddle's syndrome, a severe form of salt-sensitive arterial hypertension. The mutation prevents the binding of Nedd4–2 to the PY motif and subsequent channel ubiquitylation, retrieval and proteasomal degradation. Channel phosphorylation at positions βT613 and γT623 is thought to reduce the ability of the channel to interact with Nedd4–2 thereby reducing Nedd4–2-mediated channel retrieval. The phosphorylation site αS621 has been shown to be critical for rapid ENaC activation by recombinant SGK1 in outside-out patches. The differential role of the various phosphorylation sites remains to be determined. Additional regulatory proteins are likely to be associated with ENaC and may be co-assembled in so called lipid rafts. However, the association of ENaC with lipid rafts is still a matter of debate

cholesterol-rich micro-domains, so called lipid rafts [309], may serve as assembly platforms to facilitate the trafficking of ENaC and its functional association with regulatory proteins, e.g. with Nedd4 (see below) which may also be localised in lipid rafts [255]. Indeed, there is some evidence that in A6 renal epithelial cells ENaC is present in lipid rafts [131, 132] and that ENaC function is modified by its lipid environment [13, 349] and by removing cholesterol from the plasma membrane [15, 354]. However, at present, the localisation of ENaC within lipid rafts and the role of the lipid environment for ENaC function and regulation are still a matter of debate [126]. In this context, it is interesting to note that phosphatidylinositides have been reported to play an important role in the regulation of ENaC (for recent review, see [260]). Both phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) are thought to be permissive for ENaC activity, and increased levels of PIP_2 or PIP_3 in the membrane are associated with an increase in ENaC activity [180, 256, 258, 329, 366]. Recently, it has been reported that resting levels of PIP_2 and PIP_3 in the apical membrane of collecting duct cells affect basal ENaC activity [261, 317]. Thus, a wide range of signalling pathways influencing PIP_2 or PIP_3 levels may contribute to ENaC regulation. Attempts to identify putative PIP_2 and PIP_3 binding sites within the β- and γ-subunit of ENaC have yielded interesting but controversial results [180, 256, 258]. Moreover, it is not yet clear whether PIP_2 and PIP_3 enhance the open probability of ENaC by directly binding to the channel or by binding to closely associated proteins [260].

There is good evidence that in the kidney, in vivo long-term regulation of ENaC activity is mainly achieved by changes in the cell surface abundance of the channel [47, 280, 313]. The number of ENaC channels in the luminal membrane is the net result of synthesis and exocytotic delivery to the cell surface and of the endocytotic retrieval of channels from the luminal membrane. Endocytosed proteins either recycle back via recycling endosomes or will become degraded via lysosomal or proteasomal pathways. The cellular and molecular mechanisms that control these key steps of ENaC trafficking begin to be elucidated. One of the best studied ENaC regulatory proteins is the ubiquitin-protein ligase Nedd4–2. The C-termini of all three ENaC subunits (αβγ) contain a proline-rich PPXY (PY) motif, which is believed to be important for the interaction with Nedd4–2, promoting the ubiquitylation, endocytosis and proteasomal degradation of the channel [318]. Studies in *Xenopus laevis* oocytes and polarised MDCK renal epithelial cells have demonstrated that Liddle's syndrome mutations and/or deletions of the PY motif in β- or γENaC reduce the endocytic retrieval of ENaC from the membrane [1, 118, 204] probably by interfering with Nedd4–2-dependent ubiquitylation and subsequent clathrin-dependent endocytosis [204]. This results in an increase in the number of ENaC channels in the membrane. In addition, Liddle's syndrome mutations have been shown to increase channel open probability [7, 99], to enhance proteolytic channel cleavage [171] and to reduce Na^+ feedback inhibition of ENaC expressed in *Xenopus laevis* oocytes [161]. Thus, the functional consequences of an altered interaction between Nedd4–2 and ENaC in Liddle's syndrome are not limited to an inhibition of Nedd4–2-mediated channel retrieval from the apical plasma membrane. This indicates that this interaction is highly relevant for many aspects of ENaC regulation. Therefore, it is not surprising that other regulatory mechanisms affect ENaC activity by modifying the ability of Nedd4–2 to functionally interact with ENaC. As described above, phosphorylation of Nedd4–2 by Sgk1 [70, 311] or PKA [25, 312] blocks its ability to ubiquitylate ENaC and increases apical membrane channel density by reducing its endocytosis. Members of 14-3-3 protein family are thought to participate in this regulatory process through

direct interaction with the phosphorylated form of Nedd4–2. By maintaining Nedd4–2 in an inactive phosphorylated state, 14-3-3 proteins appear to modulate the cell-surface density of ENaC cooperatively with Sgk1 kinase [24, 144, 229]. Aldosterone selectively increases the expression of particular 14-3-3 isoforms [192], and association of these isoforms as heterodimers with phospho-Nedd4–2 appears to be required for Na^+ transport stimulation [192]. N4WBP5A is another potential ENaC regulatory protein that stimulates ENaC currents and surface expression probably by binding to the WW domains of Nedd4–2 thereby preventing their interaction with the PY motifs of ENaC [175]. Deubiquitylating enzymes (DUBs) represent an additional regulatory mechanism to counteract Nedd4–2-mediated ENaC inhibition [338]. Indeed, it has been shown that an aldosterone induced ubiquitin-specific protease, Usp2-45, deubiquitylates ENaC and stimulates ENaC-mediated Na^+ transport in cultured mpkCCD cells and in *Xenopus laevis* oocytes [91]. A recent report demonstrated that Usp2-45 deubiquitylation of ENaC not only prevents ENaC retrieval from the plasma membrane but also promotes proteolytic channel activation [289]. Interestingly, a vasopressin-inducible ubiquitin-specific protease 10 was reported to increase ENaC cell surface expression not by deubiquitylation of ENaC but by deubiquitylating and stabilising sorting nexin 3, a protein thought to promote ENaC trafficking to the plasma membrane [41]. Recently, Butterworth and co-workers used a chemical probe approach to identify deubiquitinylating enzymes active in the cortical collecting duct cell line mpkCCD [46]. One of the isolated DUBs was identified as the ubiquitin C-terminal hydrolase (UCH) isoform L3 (UCH-L3) that turned out to be a predominant DUB in endosomal compartments of the CCD cells. Pharmacological inhibition and siRNA-mediated knockdown of UCH-L3 increased ENaC ubiquitylation and reduced the activity and cell surface density of the channel at the plasma membrane [46]. Whether UCH-L3 is constitutively active or whether its function is regulated remains to be elucidated. That ubiquitylation of ENaC is relevant for its function *in vivo* was evidenced by the recent development of a Nedd4–2-deficient mouse model. Consistent with the proposed role of Nedd4–2, Nedd4–2-deficient mice show a salt-sensitive arterial hypertension that can be effectively treated with amiloride [305]. Likewise, genetic analysis provided some evidence that Nedd4–2 variants and polymorphisms are associated with salt sensitivity of blood pressure variations in humans [10, 66, 93]. Moreover, one naturally occurring human Nedd4–2 polymorphism was characterised in the oocyte expression system and found to have reduced ENaC inhibitory effects probably due to enhanced phosphorylation [102].

The Nedd4–2/ubiquitylation pathway is an important but not the only mechanism by which ENaC activity is regulated. That other mechanisms interfere with the regulation of ENaC is already indicated by the observation that the hormonal stimulation of ENaC remains preserved when the interaction between ENaC and Nedd4–2 is compromised. In mpkCCD cells, channels with mutated PY motifs in both β- and γENaC subunits still respond to aldosterone and vasopressin [12]. Likewise, in a transgenic mouse model with Liddle's syndrome, the responsiveness of ENaC to aldosterone is not only preserved but even enhanced in the renal collecting duct [67] and in the colon [22]. These latter findings are consistent with the early observation that the mineralocorticoid response was fully conserved in one of the patients with hereditary pseudohypoaldosteronism originally described by Dr. Liddle [193]. In the last few years, several other ENaC-associated proteins regulating the function of the channel have been described [117, 284, 286]. Data derived from heterologous expression systems suggest that the aldosterone-induced protein NDRG2 [355], the GILZ [26, 315, 316], K-Ras2 [215], the SNARE protein syntaxin 1a [61, 132, 263, 295], the SNARE-binding protein complexin [45] and the heat shock-induced proteins Hsc70 and Hsp70 [115, 340] contribute to the control of ENaC trafficking and activity. Experiments on the colonic epithelial HT-29 cell line indicated that the Ras-related Rab GTPases interfere with ENaC trafficking as well. Immunoprecipitations showed that ENaC interacts with Rab3 and Rab27a [294]. Over-expression of these Rabs reduced ENaC activity by reducing the cell surface expression of the channel [294]. Introduction of isoform-specific small inhibitory RNA reversed the inhibitory effect of the over-expressed Rab proteins [294, 296, 297]. Moreover, recent experiments in CHO cells transfected with ENaC and Rab proteins indicated that Rab11a co-localises with ENaC at intracellular sites and participates to the exchange of ENaC subunits from an intracellular storage pool to the plasma membrane [159]. However, the precise regulatory mechanism and physiological role of these proteins in the context of ENaC regulation remain to be established (Fig. 4).

There are numerous reports about a functional interaction of ENaC and the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel [21, 179]. In the lungs of cystic fibrosis (CF) patients, the failure of defective CFTR to inhibit ENaC is thought to cause hyperabsorption of Na^+ and fluid possibly contributing to the formation of dry sticky mucus, a hallmark of pulmonary CF pathophysiology [40, 80]. The finding that airway-specific over-expression of ENaC produces cystic fibrosis-like lung disease in mice [209] and that the symptoms can be prevented by amiloride therapy [370] supports the concept that increased ENaC activity may contribute to CF pathophysiology. Recombinant expression studies [179, 319] have shown ENaC to be inhibited by cAMP-dependent activation of CFTR, and

similar observations have been made in various epithelial tissues including mouse renal CCD cells [189]. In the ASDN, CFTR-mediated release of ATP [290] may lead to paracrine inhibition of ENaC via the stimulation of purinergic receptors [65, 188, 259]. The recent finding that mice lacking P2Y2 receptors have salt-sensitive hypertension suggests that tonic purinergic inhibition of renal ENaC may be physiologically relevant [276]. However, the molecular mechanism and physiological relevance of a regulatory relationship between ENaC and CFTR remain a subject of considerable controversy and may vary in different tissues [173, 176, 179, 230, 266]. The complexity of the ENaC–CFTR relationship is further demonstrated by studies that suggest that ENaC may have a stimulatory effect on CFTR activity [56, 147, 151] possibly by stabilising ENaC at the apical cell surface [203]. Furthermore, in *Xenopus laevis* oocytes, ENaC upregulates the renal potassium channel Kir1.1 (ROMK) in a CFTR-dependent manner [174]. CFTR is abundantly expressed in the kidney [64] and renal collecting duct cells [73, 189, 327]. Nevertheless, findings from cultured cells and heterologous expression systems have to be interpreted with caution and need to be confirmed in native renal tissue. In salt-restricted mice [163], the natriuresis induced by amiloride was shown to be significantly greater in CF mice than in wild-type controls, consistent with an increased renal ENaC activity in CF animals. However, renal abnormalities reported in CF patients are subtle [356] and a physiological role of CFTR in renal ENaC regulation remains to be demonstrated.

Conclusion and perspectives

As outlined in this review, the CNT is a major site for the fine tuning of renal Na^+ excretion and hence for the maintenance of Na^+ balance and the long-term control of arterial blood pressure. The rate-limiting step for Na^+ absorption in the CNT is ENaC. The importance of the CNT for ENaC-mediated Na^+ absorption has previously been under-estimated possibly because the CNT is relatively short and poorly accessible for in vitro studies. Indeed, most of the studies on ENaC and its regulation in native renal tissue have been performed in the collecting duct rather than in the CNT. However, the CNTs collectively comprise approximately 8% of tubular mass in the renal cortex and account for up to 10% of the overall renal Na^+ absorption. The physiological relevance of ENaC-mediated Na^+ transport in the CNT is evidenced by the observation that the collecting duct-specific deletion of αENaC in mice is fully compensated by the residual activity of ENaC in the CNT (and in the DCT2). ENaC activity has an axial gradient along the nephron with the highest activity in the CNT and with a gradual decline along the collecting duct. Indeed, in animals maintained on a standard diet, the only nephron segment with constitutive ENaC activity is probably the CNT and in some species the DCT2. Thus, under normal physiological conditions, the CNT is likely to be the primary site of aldosterone-mediated regulation of ENaC activity to respond to small changes in dietary Na^+ intake with corresponding changes in renal Na^+ excretion. Hence, the CNT probably is the major site of action for diuretics such as amiloride or aldosterone antagonists. So far, little is known about the mechanisms that establish the gradient of ENaC expression along the nephron and that specifically regulate ENaC activity in the CNT. However, it may well be relevant to understand these mechanisms since they may be involved in ENaC dysregulation under pathophysiological conditions and may contribute to states of renal Na^+ retention and the development of arterial hypertension. In this context, it will be an important challenge for future studies to characterise nephron-specific mechanisms of ENaC regulation. The axial gradient of Nedd4–2 expression along the CNT and CD may be one of the reasons for the different cell surface activity of ENaC along the ASDN. Other channel-associated proteins, the subunit composition of the channel, the expression profile of kinases and phosphatases, the lipid environment or the activation of ENaC by nephron specific proteases may contribute to a differential regulation of ENaC along the nephron. A better understanding of these factors may lead to the identification of new therapeutic targets and new perspectives for the diagnosis and treatment of renal Na^+ retention and salt-sensitive arterial hypertension.

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